Neuro-Oncology

Scatter factor/hepatocyte growth factor in brain tumor growth and angiogenesis[1]

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The multifunctional growth factor scatter factor/hepatocyte growth factor (SF/HGF) and its receptor tyrosine kinase c-Met have emerged as key determinants of brain tumor growth and angiogenesis. SF/HGF and c-Met are expressed in brain tumors, the expression levels frequently correlating with tumor grade, tumor blood vessel density, and poor prognosis. Overexpression of SF/HGF and/or c-Met in brain tumor cells enhances their tumorigenicity, tumor growth, and tumor-associated angiogenesis. Conversely, inhibition of SF/HGF and c-Met in experimental tumor xenografts leads to inhibition of tumor growth and tumor angiogenesis. SF/HGF is expressed and secreted mainly by tumor cells and acts on c-Met receptors that are expressed in tumor cells and vascular endothelial cells. Activation of c-Met leads to induction of proliferation, migration, and invasion and to inhibition of apoptosis in tumor cells as well as in tumor vascular endothelial cells. Activation of tumor endothelial c-Met also induces extracellular matrix degradation, tubule formation, and angiogenesis in vivo. SF/HGF induces brain tumor angiogenesis directly through only partly known mechanisms and indirectly by regulating other angiogenic pathways such as VEGF. Different approaches to inhibiting SF/HGF and c-Met have been recently developed. These include receptor antagonism with SF/HGF fragments such as NK4, SF/HGF, and c-Met expression inhibition with U1snRNA/ribozymes; competitive ligand binding with soluble Met receptors; neutralizing antibodies to SF/HGF; and small molecular tyrosine kinase inhibitors. Use of these inhibitors in experimental tumor models leads to inhibition of tumor growth and angiogenesis. In this review, we summarize current knowledge of how the SF/HGF:c-Met pathway contributes to brain tumor malignancy with a focus on glioma angiogenesis. *Neuro-Oncology 7, 436–51, 2005 (Posted to Neuro-Oncology [serial online], Doc. 05-005, August 10, 2005. URL http://neuro-oncology.mc.duke.edu; DOI: 10.1215/S1152851705000050)*

Keywords: c-Met, glioma, blood vessel formation

General Characteristics of SF/HGF and c-Met

Scatter factor (SF),[3] also known as hepatocyte growth factor (HGF), is a widely expressed multifunctional growth and angiogenic factor. The proto-oncogene product receptor tyrosine kinase c-Met is the only known receptor for SF/HGF. Hepatocyte growth factor was originally purified and characterized as a potent mitogen of primary cultured hepatocytes in 1984 (Nakamura et al., 1984). A fibroblast-derived epithelial cell motility factor, termed *scatter factor*, was identified in 1985 (Stoker and Perryman, 1985). Subsequent characterization revealed SF to be identical to HGF (Furlong et al., 1991; Weidner et al., 1989). c-Met was cloned in 1984 and later identified as the receptor for SF/HGF (Bottaro et al., 1991; Cooper et al., 1984).

Received January 12, 2005; accepted March 16, 2005.

[1]This research was supported by NIH RO1 Grants NS032148 and NS 43987 (John Laterra) and NIH RO1 Grant NS045209 and Children's Cancer Foundation Grant (Roger Abounader).

An application has been submitted for a U.S. patent entitled Methods of Treating Brain Tumors with Antibodies, by John J. Laterra, at the Kennedy Krieger Research Institute in Baltimore.

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[3]Abbreviations used are as follows: bFGF, basic fibroblast growth factor; SF, scatter factor; HGF, hepatocyte growth factor; HUVEC, human umbilical vascular endothelial cell; mAb, monoclonal antibody; TEM, tumor endothelial marker; VE, vascular endothelial; VEGF, vascular endothelial growth factor.

Structure of SF/HGF and c-Met

The gene encoding SF/HGF is located on chromosome 7q21.1 (Seki et al., 1991). It produces a single-chain inactive precursor that is cleaved by serine proteases into two chains that are linked by a disulfide bond (Miyazawa et al., 1993). The biologically active SF/HGF is a heterodimer composed of a 69-kDa β-chain and a 34-kDa β-chain (Nakamura et al., 1989). The α-chain contains an N-terminal hairpin domain followed by four-kringle domains, and the β-chain contains a serine protease–like domain with no enzymatic activity (Fig. 1). The human c-Met gene is located on chromosome 7q21-q31. c-Met is synthesized as a 170-kDa glycosylated precursor that is cleaved into a 50-kDa α-chain and a 140-kDa β-chain that are linked by a disulfide bridge. The α-chain is located extracellularly, where along with the first 212 residues of the β-chain, it binds to SF/HGF. The β-chain traverses the membrane and contains the cytoplasmic kinase domain and the carboxy-terminal that is essential for downstream signaling (Birchmeier et al., 2003; Gherardi et al., 2003). It also contains the c-Met docking site, which comprises tyrosines Y1349 and Y1356, phosphorylation sites for the c-Met kinase (Fig. 1) (Ponzetto et al., 1994).

c-Met-Dependent Signal Transduction

Phosphorylation of tyrosines Y1349 and Y1356 results in the recruitment and binding of numerous substrates, including Gab1, Grb2, PI3K, and others (Ponzetto et al., 1994). This leads to the activation of downstream signaling pathways including Ras/MAPK, PI3K/Akt, and STAT pathways, which mediate various functions of SF/HGF. Activation of Ras and of ERK/MAPK causes changes in the expression/activation of cell cycle regulators (including p27, cdk2, pRb, and others) leading to changes in cell proliferation. Ras/MAPK activation by SF/HGF also leads to changes in gene expression of matrix metalloproteinases and urokinase plasminogen activator and in alterations of cytoskeletal functions that control cell migration and invasion. PI3K/Akt activation by SF/HGF mediates cell survival and resistance to apoptosis through multiple mediators, including inhibition of caspase-9 and Bad. Both Ras/MAPK activation and PI3K/Akt activation are required for the complex phenomenon of tubule formation induced by c-Met activation (Birchmeier et al., 2003). The STAT signaling pathway has been implicated in epithelial tubule morphogenesis and in endothelial cell proliferation (Fig. 2) (Boccaccio et al., 1998; Nakagami et al., 2001).

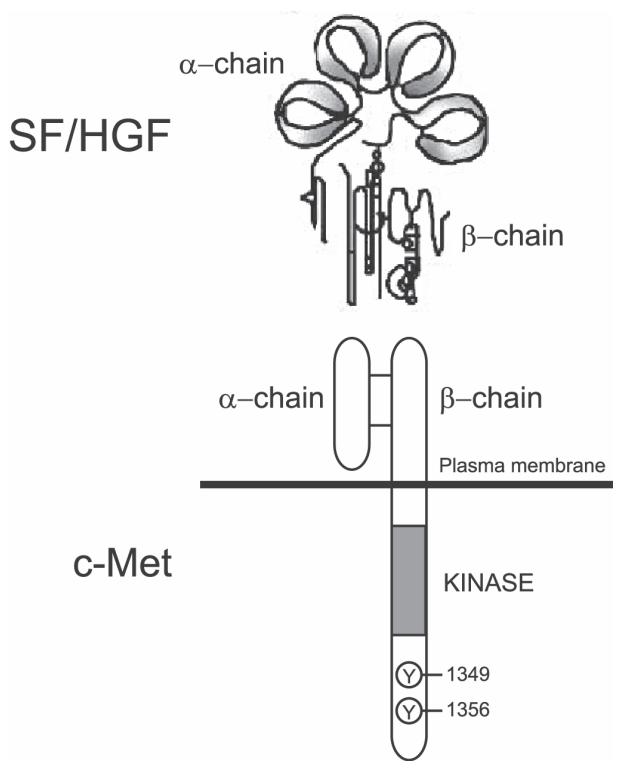

Fig. 1. Structure of SF/HGF and c-Met (modified, with permission, from Fig. 1 in Matsumoto and Nakamura, *Cancer Science* 94, 322, 2003).

Biological Functions

SF/HGF and c-Met play essential roles in embryogenesis and organogenesis (Birchmeier and Gherardi, 1998). Mice lacking SF/HGF develop severely impaired placentas and livers and die in utero (Schmidt et al., 1995; Uehara et al., 1995). SF/HGF regulates various developmental processes by mediating epithelial-mesenchymal interactions. During development, c-Met is expressed in epithelial cells in many organs, and SF/HGF is produced by adjacent mesenchymal cells (Sonnenberg et al., 1993). Exchange of signals between the mesenchymal and epithelial cell compartments has long been recognized as a major driving force in epithelial growth, morphogenesis, and differentiation. In adult tissues, SF/HGF and c-Met have been implicated in tissue regeneration and wound healing. SF/HGF and c-Met expression are upregulated in several injured organs including liver, kidney, and heart, where they promote cell proliferation and migration and inhibit cell death (Michalopoulos and DeFrances 1997).

SF/HGF and c-Met in Human Cancer

Although the effects of SF/HGF in neoplastic tissues are complex and can vary according to tissue type, cell type, and other conditions, they are generally consistent with an increased malignant phenotype in numerous human cancer models. SF/HGF and c-Met are expressed in a wide variety of human tumors, and their expression levels frequently correlate with poor prognosis (Birchmeier et al., 2003) (Table 1). The c-Met gene is amplified in some human tumors (Kuniyasu et al., 1992; Muleris et al., 1994). Overexpression of SF/HGF in animal tumor models leads to increased tumor growth and malignancy. Additionally, activating germline mutations of c-Met have been found in some cancers such as hereditary renal papillary carcinoma, and transgenic models confirm that activating c-Met mutations are oncogenic (Jeffers et al., 1997; Schmidt et al., 1997). Importantly,

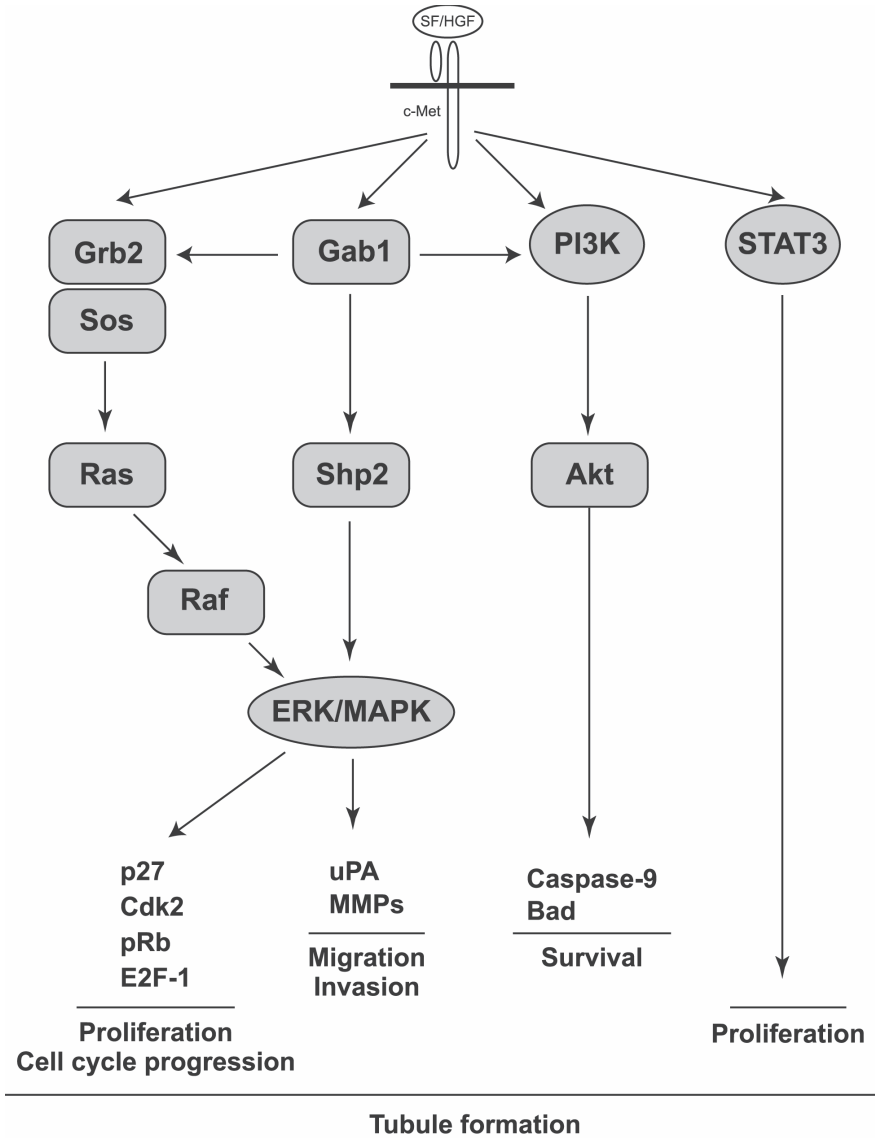

Fig. 2. c-Met-dependent signal transduction pathways, transcriptional events, and corresponding functional consequences (modified, with permission, from Fig. 6 in Birchmeier et al., *Nature Reviews Molecular Cell Biology* 4, 921, 2003).

also, downregulation of SF/HGF or c-Met in human tumor xenografts leads to inhibition of tumor growth. At the cellular level, SF/HGF exerts multiple autocrine and paracrine oncogenic actions. SF/HGF induces tumor cell proliferation and mitogenicity and allows cells to overcome G_0/G_1 arrest. SF/HGF is a potent survival factor that protects both neoplastic and tumor endothelial cells against apoptosis and cell death. SF/HGF also induces tumor cell migration and scattering and stimulates invasion and metastasis. SF/HGF plays an important role in endothelial cell migration, proliferation, and tubule formation, processes that are required for blood vessel formation.

SF/HGF and c-Met in Brain Tumors

As for human tumors in general, the implication of SF/HGF:c-Met signaling in glioma malignancy is based on the following findings: (1) SF/HGF and c-Met are expressed in human gliomas with expression levels correlating with tumor grade (Koochekpour et al., 1997; Moriyama et al., 1998a; Rosen et al., 1996), (2) gene transfer of SF/HGF to glioma cells enhances their tumorigenicity, tumor growth, and tumor-associated angiogenesis (Laterra et al., 1997a, b), and (3) inhibition of SF/HGF or c-Met expression leads to inhibition of in vivo tumor formation and growth (Abounader et al., 1999, 2002). Our group has measured the SF/HGF content in 74 clinical samples obtained from human low-grade and high-grade gliomas. We found that SF/HGF expression in high-grade (III–IV) tumors was significantly higher (3-fold; $P < 0.03$) than in low-grade (I–II) tumors (Table 2) (Lamszus et al., 1998). c-Met receptor expression has also been detected in all glioma, medulloblastoma, ependymoma, and schwannoma tumors and cell lines as well as in neuroblastoma cells examined

Table 1. SF/HGF and c-Met expression and mutations in human tumors and their correlation with prognosis*

Category	Cancer type	HGF/SF expression	Met expression	Poor prognosis	Mutation of Met
Carcinomas	Bladder	Y	Y	Y	N
	Breast	Y	Y	Y	N
	Cervical	N	Y	Y	N
	Cholangiocarcinoma	N	N	N	N
	Colorectal	Y	Y	N	N
	Esophageal	N	Y	N	N
	Gastric	Y	Y	Y	Y
	Head and Neck	Y	Y	Y	Y
	Kidney	Y	Y	N	Y
	Liver	Y	Y	Y	Y
	Lung	Y	Y	Y	N
	Nasopharyngeal	Y	Y	Y	N
	Ovarian	N	Y	N	Y
	Pancreas/gall bladder	Y	Y	N	N
	Prostate	Y	Y	N	N
	Thyroid	Y	Y	Y	N
Musculoskeletal sarcomas	Osteosarcoma	Y	Y	N	N
	Synovial sarcoma	Y	Y	N	N
	Rhabdomyosarcoma	N	Y	N	N
Soft tissue sarcomas	MFH/fibrosarcoma	Y	Y	N	N
	Leiomyosarcoma	Y	Y	N	N
	Kaposi's sarcoma	Y	Y	N	N
Hematopoietic malignancies	Multiple myeloma	Y	Y	Y	N
	Lymphomas	Y	Y	N	N
	Adult T-cell leukemia	N	Y	N	N
	Acute myelogenous leukemia	Y	N	N	N
	Chronic myeloid leukemia	Y	N	N	N
Other neoplasms	Glioblastomas/astrocytomas	Y	Y	Y	Y
	Melanoma	Y	Y	N	N
	Mesothelioma	Y	Y	N	N
	Wilms' tumor	Y	Y	N	N

*Modified, with permission, from Table 1 in Birchmeier et al., *Nature Reviews Molecular Cell Biology* 4, 922, 2003.

to date (Hecht et al., 2004; Koochekpour et al., 1997; Moriyama et al., 1998a). Interestingly, coexpression of SF/HGF and c-Met is observed more frequently in higher grade glioblastoma than in low-grade glioma, consistent with SF/HGF:c-Met autocrine loop formation contributing to increased malignancy in these tumors (Koochekpour, 1997; Moriyama et al., 1998a). Likewise, elevated SF/HGF levels and coexpression of SF/HGF and c-Met correlate with meningioma recurrence and poor prognosis (Arrieta et al., 2002; Martinez-Rumayor et al., 2004). Additional evidence implicating the SF/HGF:c-Met pathway in brain tumorigenesis and malignant progression comes from overexpression studies. In fact, SF/HGF gene transfer to rat and human glioma and medulloblastoma cell lines strongly enhances the formation and growth of tumor xenografts derived from these cells (Fig. 3) (Abounader et al., 2004a; Laterra et al., 1997a, b). The ultimate evidence for the involvement of endogenous SF/HGF and c-Met in brain tumors was provided by loss of function experiments in which highly specific U1snRNA/ribozyme chimeric transgenes were used to inhibit in vivo expression of SF/HGF and c-Met in human glioma xenografts. These studies show that SF/HGF and c-Met expression knockdown leads to substantial inhibition of tumorigenesis and tumor growth and to prolongation of the survival of animals bearing glioblastoma xenografts (Fig. 3) (Abounader et al., 1999, 2002).

At the cellular level, the SF/HGF:c-Met pathway affects brain tumor formation and malignant progression by inducing cell cycle progression, tumor cell migration, tumor cell invasion, and tumor angiogenesis and by inhibiting tumor apoptosis. SF/HGF induces the in vitro proliferation and anchorage-independent growth of various brain tumor cell lines including those derived from glioma, medulloblastomas, and neuroblastoma (Abounader et al., 2004a; Hecht et al., 2004; Koochekpour et al., 1997; Laterra et al., 1997a,b). The mitogenic

Table 2. Expression of SF/HGF in human brain tumors and their correlation to tumor grade*

Tumor Type	SF Content (ng SF/mg protein) Mean ± SEM (N)	Median	Range
High-grade (III–IV) tumors			
Glioblastoma multiforme	1.83 ± 0.67 (27)	0.60	0.0–16.5
Anaplastic astrocytoma/malignant glioma	1.63 ± 0.89 (10)	0.77	0.26–9.5
Malignant oligodendroglioma	1.54 ± 0.56 (10)	1.16	0.01–5.6
All high grade	1.73 ± 0.43 (47)	0.72	0.0–16.5
Low-grade (I–II) tumors			
Low-grade astrocytoma/glioma	0.81 ± 0.29 (8)	0.54	0.0–2.6
Benign oligodendroglioma	0.49 ± 0.16 (16)	0.20	0.0–2.1
Ependymoma	0.32 ± 0.32 (2)	0.32	0.0–0.63
All low grade	0.58 ± 0.15 (26)	0.37	0.0–2.6
Nontumor tissue			
Temporal lobe	0.49 ± 0.12 (4	0.35	0.27–1.0

*Modified, with permission, from Table 1 in Lamszus et al., *International Journal of Cancer* 75, 21, 1998).

effects of SF/HGF on tumor cells are at least partly due to its ability to mediate G_1/S cell cycle transition. In fact, our group has shown that stimulation of human glioblastoma cells with SF/HGF allows the cells to escape G_1/G_0 arrest induced by serum withdrawal and contact inhibition (Walter et al., 2002). c-Met activation by SF/HGF alters multiple cell cycle regulators, including p27, phospho-Rb, E2F-1, and c-Myc (Walter et al., 2002). SF/HGF also induces brain tumor cell migration and invasion. SF/HGF was identified as the most potent stimulator of glioma cell migration when compared with numerous other growth factors previously associated with glioma motility (Brockmann et al., 2003a). Also, brain tumor cyst fluids and brain tumor tissue extracts containing high SF/HGF content induce the migration of glioma and endothelial cell lines, and neutralizing antibodies against SF/HGF inhibit the ability of cyst fluids to induce cell migration by ~50%, which thus implicates SF/HGF as a prominent tumor-derived migration factor (Lamszus et al., 1998). Another study showed that SF/HGF strongly induces in vitro migration and invasion of four glioma cell lines but not normal human astrocytes (Koochekpour et al., 1997). The same authors also found prominent c-Met immunostaining in the infiltrative tumor cells of anaplastic astrocytoma and glioblastoma tumor specimens. SF/HGF also contributes to brain tumor malignancy by inhibiting basal and radio/chemotherapy-induced tumor cell death and apoptosis. We have shown that c-Met receptor activation by SF/HGF protects glioblastoma cells and tumor xenografts from DNA-damaging agents, in part by activating phosphoinositol 3-kinase-dependent and Akt-dependent antiapoptotic pathways (Bowers et al., 2000). In vivo inhibition of SF/HGF and c-Met in established glioblastoma xenografts leads to induction of tumor cell apoptosis quantified in tumor cross-sections immunostained with anti-activated caspase-3 (Abounader et al., 2002). These and other studies demonstrate that, in addition to their effects on tumor angiogenesis discussed below, SF/HGF and c-Met play important and critical roles in brain tumor formation and growth by regulating various other malignancy parameters.

SF/HGF as an Angiogenic Factor

Angiogenesis, the development of new blood vessels from preexisting vasculature, accompanies the growth and malignant transformation of most neoplasms and is essential for tumor growth. Angiogenesis consists of a complex process that involves proteolysis of the pericellular and extracellular matrix to create a path for the passage of proliferating, migrating, and apoptosis-resistant endothelial cells. These endothelial cells, which originate predominantly from preexisting vessels and/or, as more recently recognized, from circulating endothelial precursor cells, subsequently form tubules with a lumen and a basement membrane to become new blood vessels. Extracellular matrix degradation as well as vascular endothelial cell proliferation, migration, survival, and tubule formation are therefore essential mechanisms of angiogenesis. SF/HGF secreted by tumor cells as well as by vascular smooth muscle cells, pericytes, and fibroblasts has been found to regulate all of the above-mentioned mechanisms through activation of c-Met receptors present on vascular endothelial cells. This section summarizes the biological effects of c-Met activation in vascular endothelial cells and what is known about the signal transduction molecules/pathways that mediate these effects.

SF/HGF and c-Met Expression As It Pertains to Angiogenesis

c-Met is expressed by tumor microvessels in vivo, and tumor expression levels frequently correlate with tumor microvessel density (Kuhnen et al., 2003; Kunkel et al., 2001; Wagatsuma et al., 1998). Additionally, systemic blood levels of SF/HGF are frequently elevated in tumor-bearing patients, and these levels correlate with

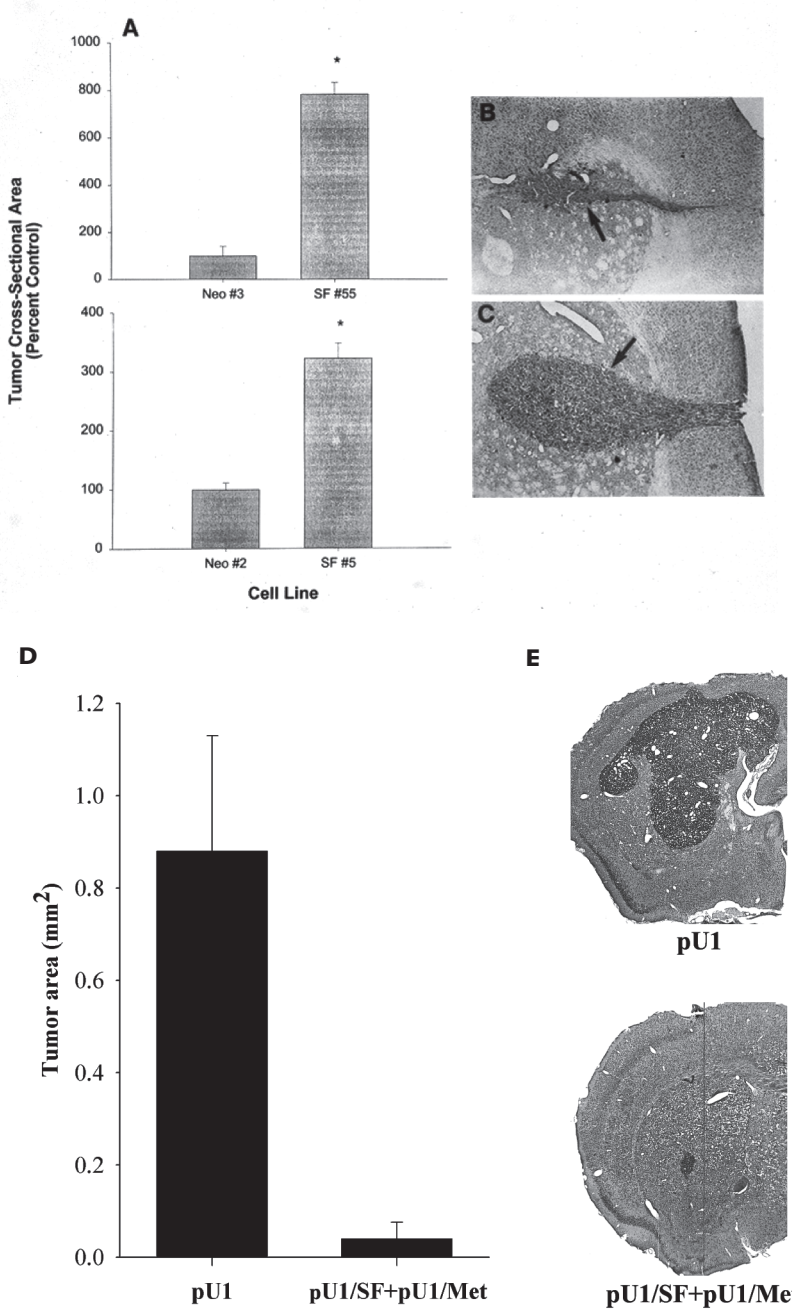

Fig. 3. SF/HGF promotes brain tumor growth in vivo. A–C. Glioma cells engineered to overexpress SF/HGF (SF #5 and SF #55) produce significantly larger xenografts (C) than control clones (A, Neo #2 and Neo #3, and B) (adapted, with permission, from Fig. 3 in Laterra et al., *Biochemical and Biophysical Research Communications* 235, 745, 1997). D–E. In vivo inhibition of SF/HGF and c-Met expression by systemic injection of liposomes complexed with plasmids expressing anti-SF/HGF U1/ribozymes (pU1/SF) and anti-c-Met U1/ribozymes (pU1/Met) leads to significant growth inhibition of wild-type intracranial glioma xenografts (pU1 = control) (modified, with permission, from Fig. 1B in Abounader et al., *FASEB Journal* 16, 109, 2002).

tumor microvessel densities (Alexandrakis et al., 2004; Skoldenberg et al., 2001). Numerous in vitro studies have shown that c-Met receptors are also expressed by cultured vascular endothelial cells (Bussolino et al., 1992; Ding et al., 2003; Nakamura et al., 1995). Vascular endothelial c-Met is active and functional as evidenced by receptor phosphorylation, activation of c-Met-dependent signal transduction pathways, and functional changes in response to SF/HGF treatment (Rosen et al., 1999). Moreover, c-Met receptor upregulation was shown to contribute to the angiogenic phenotype of human endothelial cells and to promote in vitro correlates of angiogenesis. SF/HGF is expressed and secreted by a wide variety of tumor cells and can activate endothelial c-Met receptors in a paracrine fashion. SF/HGF is also expressed and secreted by vascular smooth

muscle cells, pericytes, and fibroblasts (Hayashi et al., 1996; Martin et al., 1999; Rosen et al., 1990). Conditioned media from these cells activate c-Met and lead to functional changes in co-incubated vascular endothelial cells. One report also described SF/HGF expression in vascular endothelial cells, raising the possibility of autocrine stimulation of endothelial c-Met receptors (Nakamura et al., 1995). Therefore, the foundations for an angiogenic role of SF/HGF and c-Met in normal and neoplastic human tissues and organs are numerous and well established.

SF/HGF Enhances Vascular Matrix Degradation and Endothelial Cell Invasion and Migration

Angiogenesis requires extracellular matrix degradation and remodeling that allows pericyte and endothelial cell migration and invasion. Several studies have shown that SF/HGF accelerates matrix degradation and endothelial cell invasion. This effect is mediated, at least in part, by SF/HGF-induced expression and synthesis of matrix metalloproteinases and urokinase by vascular endothelial cells. SF/HGF enhances MT1-MMP synthesis and induces MMP-2 activation in human dermal microvessel endothelial cells and coronary arterial endothelial cell lines. Furthermore, SF/HGF accelerates endothelial cell invasion into the extracellular matrix in an MMP-dependent fashion (Oh et al., 2002; Wang and Keiser, 2000). SF/HGF also induces cultured microvascular endothelial cells to accumulate and secrete significantly increased quantities of urokinase, an enzyme closely linked to the development of an invasive endothelial phenotype during angiogenesis (Grant et al., 1993). Most of the SF/HGF-induced urokinase is bound to uPAR on the cell surface, where it is well positioned to mediate focal degradation of extracellular matrix proteins, a prerequisite for cell invasion (Rosen et al., 1999). SF/HGF has also been shown to induce other members of the plasminogen activation system in an HIF-1-dependent manner (Tacchini et al., 2003).

SF/HGF is a potent motility factor for vascular endothelial cells. Recombinant SF/HGF stimulates the chemotactic migration of neuromicrovascular endothelial cells (Lamszus et al., 1998). Fibroblasts have been shown to induce the migration of human large-vessel endothelial cells in an SF/HGF-dependent manner (Martin et al., 1999). SF/HGF also increases the dissociation and migration of human umbilical vascular endothelial cells (HUVECs). This action of SF/HGF on HUVECs occurs through regulation of the endothelial cell–specific cadherin, or vascular endothelial cadherin (Martin et al., 2001). SF/HGF-induction of endothelial cell migration in endothelial cells derived from the human saphenous vein is mediated by iNOS, a well-described endothelial cell motility factor (Purdie et al., 2002).

SF/HGF Induces Proliferation and Inhibits Apoptosis of Vascular Endothelial Cells

SF/HGF strongly induces DNA synthesis and proliferation in vascular endothelial cells of various origins, including neuromicrovascular endothelial cells and human aortic endothelial cells (Hayashi et al., 1996; Lamszus et al., 1998; Nakagami et al., 2001). Recombinant SF/HGF as well as conditioned media from vascular smooth muscle cells stimulates vascular endothelial cells to grow in an SF/HGF-dependent manner. Endothelial cell proliferation was found to require MAPK/ERK and STAT3 pathway activation (Nakagami et al., 2001). In addition to its proliferative effects, SF/HGF also enhances endothelial cell survival and renders vascular endothelial cells resistant to apoptosis and cell death induced by various conditions including serum deprivation and hypoxia (Ma et al., 2002; Nakagami et al., 2001; Wang et al., 2004; Yamamoto et al., 2001). The signal transduction pathways that mediate these cytoprotective effects are only partly known. Different mechanisms and pathways have been implicated by using a variety of conditions and cell lines. For instance, SF/HGF-induced survival of human umbilical endothelial cells is mediated by MAPK/ERK and AKT (Ma et al., 2002). SF/HGF prevents human aortic endothelial cell death induced by hypoxia in a Bcl-2-, but not a Bcl-xL- or Bax-dependent fashion (Yamamoto et al., 2001). Protection of hypoxia-induced apoptosis in mouse lung endothelial cells was associated with inhibition of p38 MAPK and Bid/Bax as well as increased expression of Bcl-xL (Wang et al., 2004).

SF/HGF Induces Endothelial Tubule Formation

Capillary-like tubule formation is an essential step in angiogenesis and is an independent property of endothelial cells not related to motility and migration (Grant et al., 1995). SF/HGF was shown to induce tubule formation by human umbilical vein endothelial cells in a dose-dependent manner (Jiang et al., 1999). SF/HGF-producing matrix-bound fibroblasts induced capillary tube formation by these cells in a Matrigel assay (BD Biosciences, San Jose, Calif.), an effect that was inhibited by anti-SF/HGF antibodies (Martin et al., 1999). Also, SF/HGF significantly increased endothelial cell tube formation in an angiogenesis co-culture assay of endothelial cells and SF/HGF-secreting keratinocytes (Wojta et al., 1999). These findings demonstrate that SF/HGF regulates all steps of blood vessel formation at the cellular level and reflect its potent and broad involvement in angiogenesis.

SF/HGF Interacts with Other Angiogenesis Regulators

The molecular mechanisms through which SF/HGF regulates angiogenesis are not fully known. Although substantial evidence demonstrates a direct role for SF/HGF at all levels of the angiogenic process, SF/HGF can also affect angiogenesis by regulating the expression levels of other well-known proangiogenic and antiangiogenic factors such as vascular endothelial growth factor (VEGF) and thrombospondin 1. SF/HGF has been shown to induce VEGF mRNA and protein expression in normal and neoplastic cells (Moriyama et al., 1998b; Wojta et al., 1999). SF/HGF was also found to induce the expres-

sion of the VEGF receptor flk-1 in an endothelial cell line (Wojta et al., 1999). Induction of VEGF by SF/HGF was shown to be mediated by MAPK, PI3K, PKC-zeta and phosphorylation of Sp1, a regulator of the VEGF promoter (Reisinger et al., 2003; Zhang et al., 2003). In another human endothelial cell line, VEGF induction by SF/HGF was dependent on the upregulation of essential transcription factor ets-1 (Tomita et al., 2003). The contribution of VEGF to SF/HGF-induced angiogenesis was found to be either additive or synergistic, depending on the cells/tissues examined. For instance, SF/HGF and VEGF had additive effects on HUVEC proliferation and synergistic effects on HUVEC migration (Van Belle et al., 1998). In other studies, SF/HGF was found to act in concert with VEGF to promote human vascular endothelial cell survival and tubulogenesis in 3-D type I collagen gels, a response that did not occur with either growth factor alone. The synergistic effects of combining VEGF and SF/HGF on endothelial survival correlated with the greatly augmented expression of the antiapoptotic genes Bcl-2 and A1 (Xin et al., 2001). SF/HGF and VEGF have also been shown to promote angiogenesis in a coculture assay by inducing distinguishable patterns of vascular morphology. VEGF was found to increase the length, area, and branch point number of induced vessels, whereas SF/HGF mediated exclusively vascular area growth, which resulted in vascular structures of enlarged diameter. Moreover, the combination of both cytokines resulted in an additive increase in vascular diameter (Beilmann et al., 2004). Consistent with these findings, gene expression profiling showed very little overlap between genes that are significantly changed by either SF/HGF or VEGF in endothelial cells (Gerritsen et al., 2003). These latest data show that the combination of SF/HGF and VEGF results in the cooperative upregulation of a number of different molecular pathways, leading to a more robust proliferative and angiogenic response. In addition to upregulating VEGF, SF/HGF was shown to simultaneously downregulate the expression of thrombospondin 1, a negative regulator of angiogenesis (Zhang et al., 2003). Besides its cooperation with VEGF, SF/HGF has been shown to induce angiogenesis independently of VEGF. In fact, SF/HGF can still induce endothelial tube formation in vitro as well as angiogenesis in vivo in the setting of VEGF inhibition (Sengupta et al., 2003).

SF/HGF Promotes In Vivo Angiogenesis

In parallel with its tumorigenic effects, numerous studies have shown that SF/HGF is a potent angiogenic factor in vivo in a wide variety of human normal tissues, tumors, and derived xenografts. Gain-of-function studies show that activating the SF/HGF:c-Met pathway enhances in vivo angiogenesis. Using two different in vivo assays, Grant et al. showed that physiologic quantities of purified native mouse SF/HGF and recombinant human SF/HGF induce in vivo angiogenesis. The angiogenic activity was blocked by specific anti-SF/HGF antibodies (Grant et al., 1993). To determine whether SF/HGF can modulate the in vivo growth of human breast cancers within a natural mammary environment, Lamszus et al. studied the orthotopic growth of SF/HGF-transfected versus control clones of MDAMB231 human mammary carcinoma cells in the mammary fat pads of athymic nude mice. SF/HGF-transfected tumors had significantly higher tumor microvessel densities than control tumors ($P < 0.001$). Moreover, there were much higher titers of chemotactic activity for microvascular endothelial cells in cell-conditioned medium and primary tumor extracts from SF/HGF-transfected clones as compared with control clones (Lamszus et al., 1997). Other studies used loss of function approaches to demonstrate the role of endogenous SF/HGF in in vivo angiogenesis. Kuba et al. showed that inhibiting SF/HGF leads to a dramatic decrease in microvessel density in mammary carcinoma xenografts (Kuba et al., 2000).

SF/HGF in Brain Tumor Angiogenesis

SF/HGF Is Expressed in Brain Endothelial Cells and Tumor Blood Vessels

Numerous studies have shown that c-Met and SF/HGF are expressed and functional in neuromicrovascular and brain tumor vascular cells. Rosen et al. found moderate to high levels of immunoreactive and biologically active SF/HGF in cultured neural microvascular endothelial cells (Rosen et al., 1996).

Using double immunofluorescence staining and quantitative confocal laser scan microscopy, Koochekpour et al. showed that the intensity of SF/HGF and c-Met staining in human primary brain tumors increases with the grade of malignancy and is prevalent in both the infiltrating tumor cells and endothelial hyperplastic areas. Additionally, SF/HGF and c-Met immunostaining was observed in the endothelial cells in perivascular and vascular areas of glioblastoma (grade IV astrocytoma), whereas less intense c-Met and SF/HGF immunostaining was observed in areas of neovascularization within low-grade astrocytoma (grade I) and anaplastic astrocytoma (grade III) (Koochekpour et al., 1997).

Kunkel et al. used in situ hybridization to analyze glioblastoma, anaplastic astrocytoma, diffuse astrocytoma, pilocytic astrocytoma, and normal brain for the expression of SF/HGF mRNA. They detected strong SF/HGF expression in the majority of the tumor cells and in vascular endothelial cells in all glioblastoma specimens analyzed. c-Met immunoreactivity was observed in GFAP-expressing astrocytic tumor cells and endothelial cells as well as in a subset of microglia/macrophages. These in vivo findings are consistent with other experimental evidence pointing to autocrine and paracrine stimulation of tumor cells and endothelium through the SF/HGF:c-Met system as a contributor to tumor growth, invasion, and angiogenesis (Kunkel et al., 2001).

SF/HGF Induces Brain Tumor Endothelial Cell Proliferation and Migration

Rosen et al. found that SF/HGF stimulates the proliferation of neuromicrovascular endothelial cells by para-

crine and autocrine mechanisms. Conditioned medium from both glioblastoma and neuromicrovascular endothelial cells contained SF-IF (SF-inducing factor) activity, defined by its ability to stimulate SF production in an indicator cell line (MRC5 human fibroblasts). This activity consisted of a high-molecular-weight (>30 kDa), heat-sensitive (presumably protein) component, and a low-molecular-weight (<30-kDa), heat-stable component. Furthermore, glioblastoma-conditioned medium stimulated neuromicrovascular endothelial cells to produce SF/HGF, and neuromicrovascular endothelial cell–conditioned medium stimulated glioblastoma cells to produce SF/HGF. The magnitude of increased SF/HGF production in response to these conditioned media was 3- to 6-fold in each case. These findings demonstrate that SF/HGF-dependent interactions between glioma cells, and between glioma cells and endothelium, can contribute to the heterogeneous proliferative and angiogenic phenotypes of malignant gliomas in vivo (Rosen et al., 1996). To model angiogenic events associated with malignant progression, our group examined angiogenic responses to 9L gliosarcoma engineered to express human SF/HGF (9L-SF). Conditioned medium from 9L-SF cells stimulated thymidine incorporation into microvessel brain endothelial cells 3- to 4-fold higher than conditioned medium from 9L-neo controls (Laterra et al., 1997a).

In another study, Lamszus et al. (1998) demonstrated that SF/HGF stimulated DNA synthesis in three of three neuromicrovascular endothelial cell lines. In line with these biologic responses to SF/HGF, four of four neuromicrovascular endothelial cell lines expressed mRNA for c-Met, the SF/HGF receptor.

Brockmann et al. found that SF/HGF stimulates motility of cerebral microvascular endothelial cells. The authors used a modified Boyden chamber to analyze the chemotactic effects of various growth factors on glioma cells and primary cultures of cerebral microvascular endothelial cells. SF/HGF significantly stimulated chemotaxis of both glioma and cerebral microvascular endothelial cells (Brockmann et al., 2003a).

SF/HGF Induces VEGF Expression in Brain Tumors

Moriyama et al. investigated the effect of SF/HGF on vascular endothelial growth factor (VEGF) expression of c-Met-positive human glioma cell lines. Treatment of the glioma cells with various concentrations of SF/HGF increased transcription of VEGF mRNA in a dose-dependent fashion and enhanced the secretion of VEGF protein. Since malignant gliomas frequently co-express SF/HGF and c-Met, these results suggest that, in addition to its direct angiogenic activities, SF/HGF can act as an indirect angiogenic factor through autocrine induction of VEGF expression and secretion in malignant gliomas (Moriyama et al., 1998b).

In another interesting study, Schmidt et al. (1999) evaluated the role of three growth factors, VEGF, SF/HGF, and basic fibroblast growth factor (bFGF), in the angiogenic cascade by determining their levels in extracts of 71 gliomas by ELISA (enzyme-linked immunosorbent assay). The levels of bFGF in gliomas of WHO grade II (low grade) were only marginally different from those in gliomas of grades III and IV (high grade). In contrast, the mean concentrations of VEGF were 11-fold higher in high-grade tumors and those of SF/HGF, 7-fold higher. Both SF/HGF and VEGF but not bFGF were highly significantly correlated with microvessel density ($P < 0.001$) as determined by immunostaining for factor VIII-related antigen (Fig. 4). The investigators concluded that SF/HGF and VEGF are independent predictive parameters for glioma microvessel density as determined by multiple regression analysis.

SF/HGF Induces Brain Endothelial Tubule Formation In Vitro and Brain Tumor Angiogenesis

Our group has shown that rat gliosarcomas derived from 9L cells transfected with human SF/HGF were more angiogenic than controls on the basis of both elevated peak (2.25-fold; $P < 0.005$) and mean (1.7-fold; $P < 0.008$) blood vessel densities (Fig. 5) (Laterra et al., 1997a). Schmidt et al. (1999) measured the capacity of bFGF, VEGF, and SF/HGF to induce endothelial tube formation in a collagen gel. bFGF was found to function as an essential cofactor with which VEGF as well as SF/HGF independently synergized. As predicted by the tissue levels of the angiogenic factors, extracts from high-grade tumors were significantly more potent in the tube formation assay than the low-grade extracts ($P = 0.02$). Adding neutralizing antibodies to either bFGF, VEGF, or SF/HGF to the extracts inhibited tube formation by up to 98%, 62% and 54%, respectively. Taken together, these in vitro and in vivo findings suggest that bFGF is required but not sufficient to induce angiogenesis in gliomas. It was proposed that bFGF, which is already elevated in poorly vascularized low-grade glioma, synergizes in high-grade glioma with rising levels of not only VEGF but also of SF/HGF (Schmidt et al., 1999). Lafleur et al. (2002) showed that macro- and microvascular endothelial cells formed tubular structures when cultured within a three-dimensional fibrin matrix, a process that is enhanced by VEGF, bFGF, and SF/HGF. Endothelial tubulogenesis is also increased in cocultures of endothelial cells and tumor cells such as U87 glioma cells that secrete angiogenic factors, including SF/HGF, but not with nontumorigenic cell types such as MDCK (Madin-Darby canine kidney) epithelial cells (MacDonald et al., 2001). Hecht et al. (2004) show that SF/HGF stimulates invasion of neuroblastoma cells in vitro and in vivo and promotes the formation of angiogenic neuroblastomas in vivo. Experimental neuroblastomas treated with exogenous SF/HGF grew beyond critical angiogenesis-dependent sizes (approximately 2–3 mm) and reached diameters of up to 15 mm, while non-treated controls rarely formed solid tumors. In addition, the SF/HGF-treated tumors were well vascularized and also harbored pathological, immature vessels, which gave rise to intratumoral hemorrhage consistent with SF/HGF-mediated angiogenesis in the neuroblastomas. Smooth muscle actin immunofluorescence within these tumors demonstrated numerous blood vessels.

The multifunctional angiogenic effects of SF/HGF are illustrated in Fig. 6.

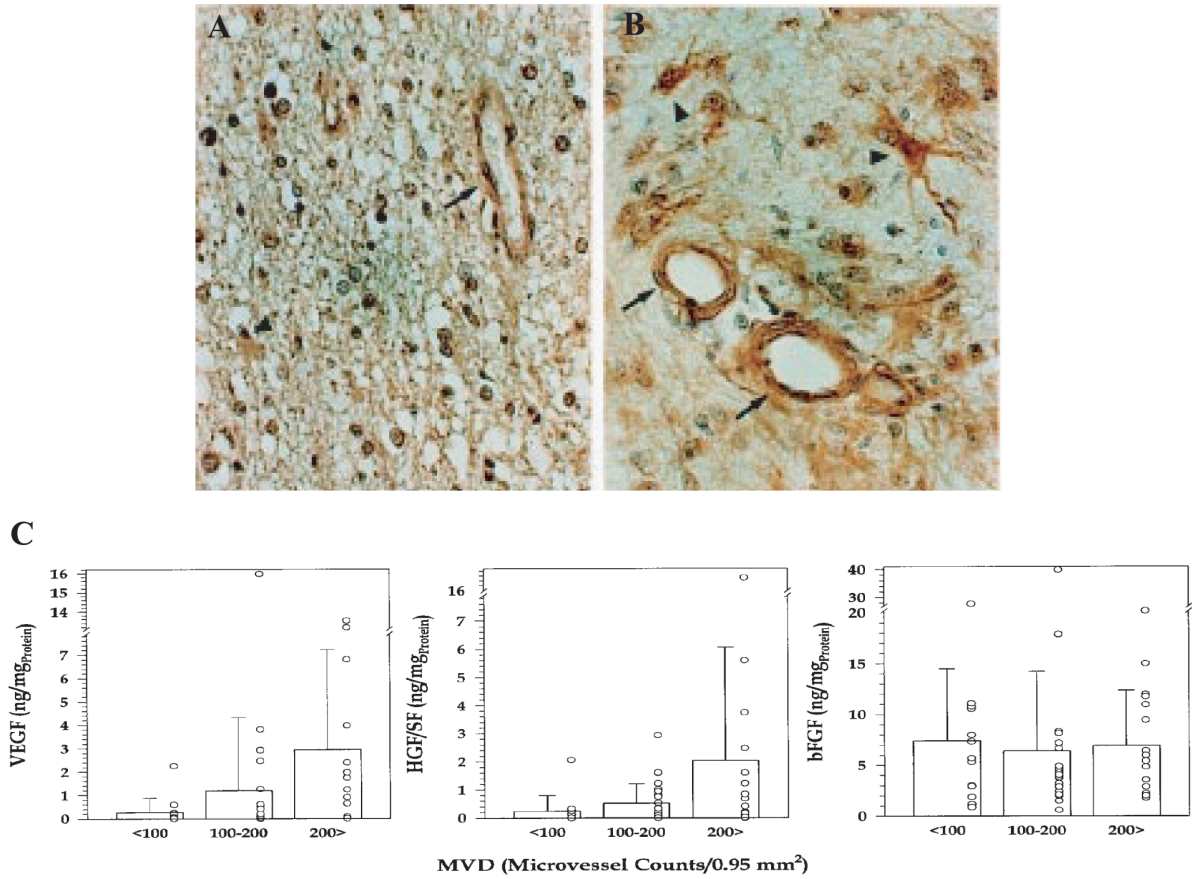

Fig. 4. SF/HGF and c-Met are expressed in human brain tumor blood vessels, and SF/HGF expression levels correlate with tumor vessel density (i.e., angiogenesis). A–B. Immunostaining of human gliomas for SF/HGF (A) and c-Met (B). Arrows indicate immunoreactivity of tumor associated-blood vessels. Arrowheads point to immunoreactive tumor cells. (Adapted, with permission, from Fig. 3 in Lamszus et al., *International Journal of Developmental* Neuroscience 17, 523, 1999.) C. Relationship between intratumoral growth factor levels and microvessel densities in human gliomas. Tumors were divided into groups with low (<100 microvessels/0.95 mm^2), medium (100–200 microvessels/0.95 mm^2), and high (>200 microvessels/0.95 mm^2) microvessel densities. VEGF and SF/HGF concentrations were statistically significantly higher in tumors with high microvessel densities than in those with low or medium microvessel densities. No such difference was detected for bFGF. (Modified, with permission, from Fig. 1 in Schmidt et al., *International Journal of Cancer* 84, 12, 1999.)

Therapeutic Considerations

The multifunctional and multilevel involvement of the SF/HGF:c-Met pathway in brain tumor angiogenesis as well as brain tumor growth demonstrates that this pathway constitutes a promising target for brain tumor therapy. In this section we review the different approaches to SF/HGF and c-Met inhibition and their potential use for future antiangiogenic and antitumorigenic therapies.

U1snRNA/Ribozymes

Our laboratory has constructed and successfully used chimeric U1snRNA/ribozyme transgenes to inhibit SF/HGF and c-Met expression in human glioma cells and xenografts (Abounader et al., 1999, 2002, 2004b). Delivery of U1snRNA/ribozymes to established subcutaneous glioma xenografts via liposome-DNA complexes significantly inhibited tumor growth as well as tumor SF/HGF and c-Met expression levels. Histologic analysis of tumors treated with U1snRNA/ribozymes showed a significant decrease in blood vessel density (Fig. 5). Recently, we have used U1snRNA/ribozyme-mediated in vivo inhibition of SF/HGF and c-Met in combination with hypofractionated γ-radiation administered every five days at 300 cGy per fraction and achieved synergistic inhibition of intracranial glioblastoma xenograft growth. Ionizing radiation therapy and U1snRNA therapy had additive effects on angiogenesis inhibition demonstrating the potential value of applying low-dose radiation in conjunction with SF/HGF pathway inhibition to target glioma blood vessel formation (Lal et al., 2005).

NK4

NK4 is a synthetic molecule that comprises the NH_2-terminal hairpin domain and subsequent four-kringle domains of SF/HGF, but lacks the entire α-chain. NK4 inhibits the specific binding of SF/HGF to its receptor c-Met in a competitive fashion and inhibits SF/HGF-

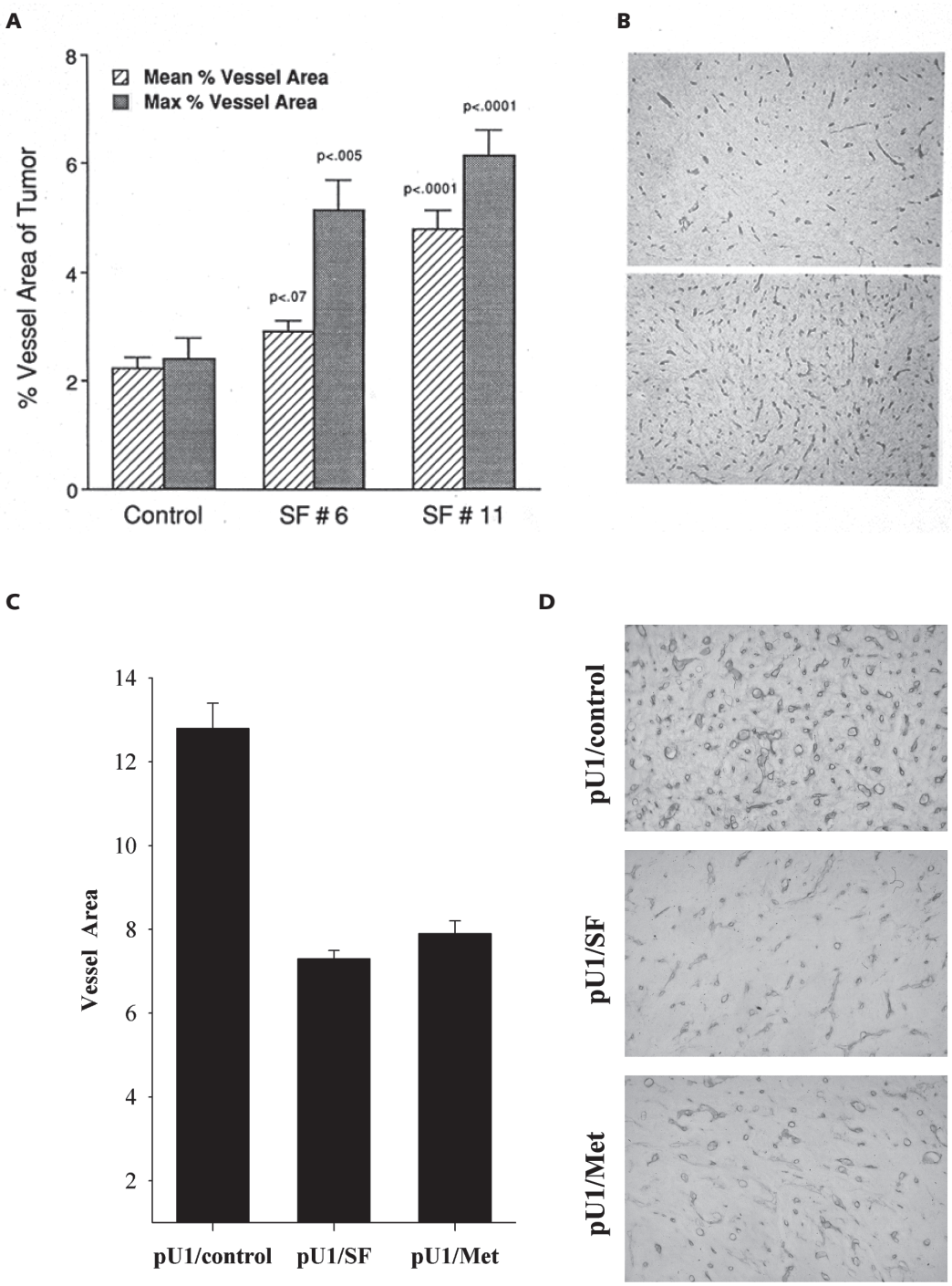

Fig. 5. SF/HGF induces angiogenesis in experimental glioblastoma. A–B. Rat glioma xenografts originating from glioma cells that overexpress SF/HGF (SF #6 and SF #11) exhibit significantly higher vessel densities than control xenografts (adapted, with permission, from Figs. 8A and B in Laterra et al., *Laboratory* Investigation 76, 572, 1997). C–D. In vivo ition of SF/HGF and c-Met expression by systemic injection of liposomes complexed with plasmids expressing anti-SF/HGF U1/ribozymes (pU1/SF) and anti-c-Met U1/ribozymes (pU1/Met) leads to a significant inhibition of tumor vessel formation (modified, with permission, from Fig. 2 in Abounader et al., *FASEB Journal* 16, 110, 2002).

induced effects on tumor cells and endothelial cells. In one study, mice bearing intracranial glioma xenografts received daily intratumoral injections of NK4 or buffer beginning on either day 1 or day 7 after tumor cell injection and continuing until day 20. Tumor volume was reduced by 61% in mice that had been treated with NK4 postimplantation, on either day 1 or day 7. Intratumoral microvessel density was reduced by 65% when treatment started on day 1 and by 37% when it started on day 7 (Brockmann et al., 2003b). The proliferative

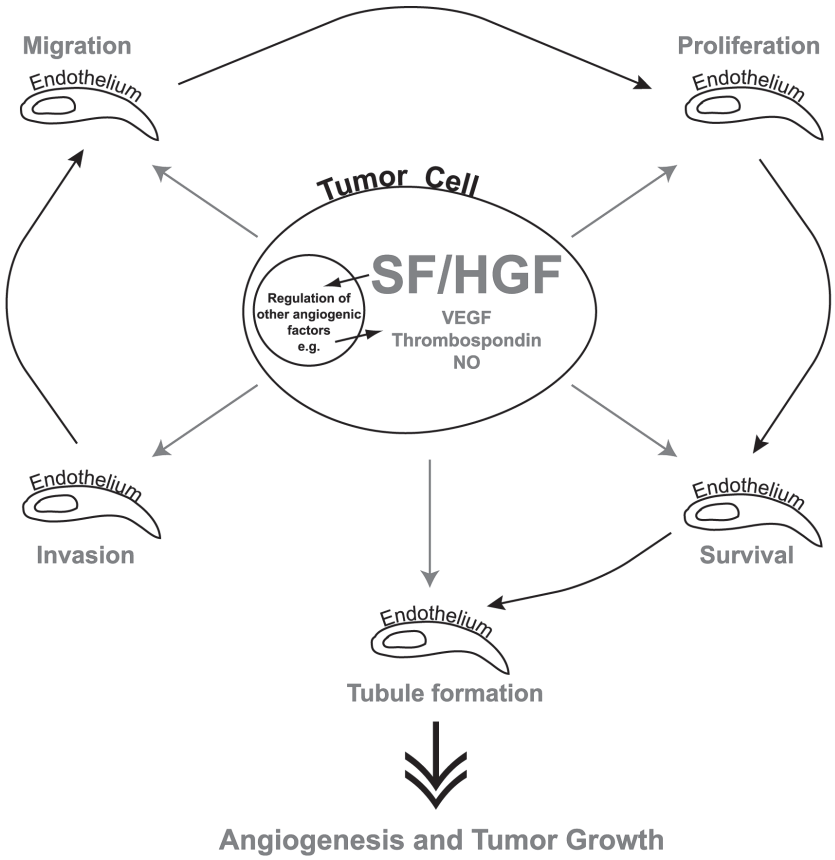

Fig. 6. Multifunctional angiogenic effects of SF/HGF.

activity of the tumor cells was reduced by >30% regardless of when NK4 treatment was initiated. The apoptotic fraction of tumor cells was increased 2-fold and 1.5-fold when animals were treated with NK4 as of day 1 or day 7, respectively. In vitro, NK4 inhibited SF/HGF-induced glioblastoma and endothelial cell migration and proliferation in a dose-dependent fashion (Brockmann, 2003b). Interestingly, NK4 is also able to inhibit angiogenesis independently of c-Met (Matsumoto and Nakamura, 2003). This effect is thought to be caused by NK4's remarkable structural homology with the antiangiogenic protein angiostatin and its potential to inhibit other kringle-dependent angiogenic protein-protein interactions (O'Reilly et al., 1994).

Soluble Met

Recently, Michieli et al. generated a soluble c-Met receptor consisting of a recombinant protein corresponding to the entire extracellular domain of c-Met, truncated before the transmembrane domain (Michieli et al., 2004). This decoy c-Met delivered via lentiviral vectors was shown to inhibit c-Met activation by SF/HGF as well as ligand-independent mechanisms in various in vitro and in vivo cancer models. The authors showed that decoy c-Met impairs SF/HGF-induced endothelial cell migration and branching. Decoy c-Met also inhibited the growth and dissemination of established mammary tumor xenografts. In these tumors, inhibition of c-Met led to inhibition of tumor vessel arborization. Using ex vivo studies, the authors subsequently showed that concomitant actions on tumor and endothelial cells by decoy c-Met result in optimal inhibition of tumor growth. Importantly, systemic administration of decoy c-Met prevented tumor growth, metastasis, and angiogenesis without substantially affecting housekeeping physiological functions of normal tissues.

Small Molecule Inhibitors

One of the most promising prospects for interfering with oncogenic SF/HGF:c-Met pathway activation arises from small molecule inhibitors of c-Met tyrosine kinase. These small molecules possess the potential for better bioavailability following systemic delivery compared to approaches dependent upon gene therapy or the delivery of larger proteins. PHA-665752 is a small-molecule, ATP-competitive, active-site inhibitor of the catalytic activity of c-Met kinase with an IC_{50} of 9 nM. PHA-665752 also exhibits >50-fold selectivity for c-Met as compared with a panel of diverse tyrosine and serine-threonine kinases including the epidermal growth factor receptor. In cellular studies in vitro, PHA-665752 potently inhibited constitutive and SF/HGF-stimulated c-Met phosphorylation, as well as SF/HGF-driven phenotypes such as cell growth (proliferation and survival),

cell motility, invasion, and/or morphology of a variety of tumor cells. In addition, PHA-665752 inhibited SF/HGF-stimulated or constitutive phosphorylation of mediators of downstream signal transduction of c-Met, including Gab-1, extracellular regulated kinase, Akt, STAT3, phospholipase C gamma, and focal adhesion kinase, in multiple cell lines. The pattern of inhibition was found to correlate with the phenotypic response of a given tumor cell. In in vivo studies, a single dose of PHA-665752 inhibited c-Met phosphorylation in tumor xenografts for up to 12 h. Inhibition of c-Met phosphorylation was associated with dose-dependent tumor growth inhibition or growth delay over a repeated administration schedule at well-tolerated doses. Potent cytoreductive activity was demonstrated in a gastric carcinoma xenograft model. Collectively, these results demonstrate the feasibility of selectively targeting c-Met with ATP-competitive small molecules and suggest the therapeutic potential of targeting c-Met in human cancers (Christensen et al., 2003).

Neutralizing Antibodies

Cao et al. developed and tested antibodies against SF/HGF. Their results showed that no single monoclonal antibody (mAb) was capable of neutralizing the in vitro activity of SF/HGF. The authors concluded that SF/HGF possesses a minimum of three epitopes that must be blocked to prevent Met tyrosine kinase activation. In vivo, in athymic nu/nu mouse, a neutralizing mAb combination inhibited subcutaneous growth of tumors that was dependent on an autocrine SF/HGF:c-Met loop. Importantly, growth of human glioblastoma multiforme xenografts expressing Met and HGF/SF was markedly reduced in the presence of SF/HGF-neutralizing mAbs (Cao et al., 2001).

Future Directions

Evidence from numerous sources points to SF/HGF being as potent or nearly as potent an endothelial mitogen, motility factor, and angiogenic factor as VEGF. Unlike VEGF, the SF/HGF receptor is also expressed on neoplastic cells, where it can potently and directly influence cancer cell behavior. As a consequence, the bulk of information regarding c-Met activation by SF/HGF, subsequent cell signaling events, and transcriptional effects has been derived from experiments using tumor cells as opposed to endothelial cells and other vascular cells. The biochemistry of how c-Met selectively activates PI3-kinase/AKT, Ras/MAP-kinase, Stat transcription factors, and other pathways is complex, cell-type specific, and contextual. It is necessary to define how these responses are regulated within vascular cells, including endothelial progenitors involved in tumor-associated vasculogenesis, and co-modulated by other angiogenesis regulators. Such mechanistic details will likely identify particularly important nodes of signal pathway activation and be useful for designing and using novel antiangiogenic cancer therapies.

Vascular cells that participate in tumor-associated neovascularization express unique antigens or tumor endothelial markers (TEMs) (Madden et al., 2004; St. Croix et al., 2000;). TEMs can serve as biomarkers of tumor angiogenesis, and they may ultimately be useful as antigenic or mechanistic targets for antiangiogenic therapeutics. It is possible that the neovasculature of highly heterogeneous neoplasms such as glioblastoma multiforme will express specific patterns of TEM expression based upon the predominant angiogenic factor(s) (e.g., SF/HGF vs. VEGF) driving the angiogenic response. Such information if obtainable via histopathological analysis would be invaluable for designing tumor-specific antiangiogenic therapeutics. In this context, avenues of investigation designed to distinguish how vascular cells differentially respond at the transcriptional and translational levels to SF/HGF and other angiogenic factors will be particularly relevant.

Very strong correlative and experimental data warrants the aggressive development and testing of therapeutic agents directed at the potently tumor-promoting and angiogenic SF/HGF:c-Met pathway. The development of specific and clinically translatable c-Met inhibitors has lagged behind efforts directed at other receptor tyrosine kinases (e.g., EGFR, Trk-A, PDGFR, and FLT-1) despite serious efforts from numerous pharmaceutical and biotech research and development teams. The fact that there are hundreds of thousands of patients with common systemic cancers in addition to patients with glioma who may benefit from these drugs all but ensures that these efforts will continue until successful. Recent approaches for inhibiting SF/HGF and c-Met gene expression and neutralizing anti-SF/HGF antibodies with potent in vivo preclinical antiglioma activity predict that clinically translatable agents are on the horizon. Existing preclinical data indicates that while anti-SF/HGF:c-Met pathway strategies will have activity against malignant glioma as a single agent, more durable cytotoxic responses may require concurrent cytotoxic therapy. This reflects cytoprotective effects of c-Met activation in glioma cells and the well-documented ability of antiangiogenic agents to synergize with cytotoxic agents. Thus, adequate clinical testing of anti-SF/HGF:c-Met treatment strategies will require early applications in newly diagnosed patients concurrent with existing standard radiation or radiation plus chemotherapy regimens.

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